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Subcellular proteomic analysis of host-pathogen interactions using human monocytes exposed to *Yersinia pestis* and *Yersinia pseudotuberculosis*

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Abbreviations:

The abbreviations used are: BVA, biological variation analysis; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Cy2, 3-([4-carboxymethyl] phenylmethyl)-3'-ethyloxacarbocyanine halide N-hydroxysuccinimidyl ester; Cy3, 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide N-hydroxysuccinimidyl ester; Cy5, 1-(5-carboxypentyl)-1'-methylindodicarbocyanine halide N-hydroxysuccinimidyl ester; DIA, differential in-gel analysis; DIGE, Differential in Gel Electrophoresis; FBS, fetal bovine serum; Yops, Yersinia Outer Proteins.

Keywords: host response / subcellular proteomics / 2-D DIGE / biodefense / biomarkers / presymptomatic / *Yersinia*

Summary

Yersinia pestis, the etiological agent of plague, is of concern to human health both from an infectious disease and a civilian biodefense perspective. While Y. *pestis* and *Y. pseudotuberculosis* share more than 90% DNA homology, they have significantly different clinical manifestations. Plague is often fatal if untreated, yet *Y. pseudotuberculosis* causes severe intestinal distress and is rarely fatal. A better understanding of host response to these closely related pathogens may help explain the different mechanisms of virulence and pathogenesis that result in such different clinical outcomes. The aim of this study was to characterize host protein expression changes in human monocyte-like U937 cells after exposure to *Y. pestis* and *Y. pseudotuberculosis*. In order to gain global proteomic coverage of host response, proteins from cytoplasmic, nuclear and membrane fractions of host cells were studied by 2-dimensional differential gel electrophoresis (2-D DIGE) and relative protein expression differences were quantitated. Differentially expressed proteins, with at least 1.5 fold expression changes and *p* values of 0.01 or less, were identified by MALDI-MS or LC/MS/MS. With these criteria, differential expression was detected in 16 human proteins after *Y. pestis* exposure and 13 human proteins after *Y. pseudotuberculosis* exposure, of which only two of the differentially expressed proteins identified were shared between the two exposures. Proteins identified in this study are reported to be involved in a wide spectrum of cellular functions and host defense mechanisms including apoptosis, cytoskeletal rearrangement, protein synthesis and degradation, DNA replication and transcription, metabolism, protein folding, and cell signaling. Notably, the differential expression patterns observed can distinguish the two pathogen exposures from each other and from unexposed host cells. The functions of the differentially expressed proteins

identified provide insight on the different virulence and pathogenic mechanisms of *Y. pestis* and *Y. pseudotuberculosis*.

Introduction

There are eleven species in the genus *Yersinia*, three of which are pathogenic to humans including *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* (1, 2). *Y. pestis* is the etiological agent of plague, which in the pneumonic form, is highly contagious and often rapidly fatal without prompt diagnosis and antibiotic intervention. *Y. pseudotuberculosis* and *Y. enterocolitica,* however, cause only gastrointestinal disorders in humans and are rarely fatal. Notably, *Y. pestis* was responsible for three devastating pandemics throughout history including the Black Death of Europe in the 14th century. It has been proposed that *Y. pestis* evolved from *Y. pseudotuberculosis* shortly before the first known pandemics of human plague, and the two pathogens share more than 90% DNA homology (3).

Both *Y. pestis* and *Y. pseudotuberculosis* function via a Type III secretion mechanism whereby virulence factors, or Yops (Yersinia outer proteins), are injected into host cells (4). It has been previously demonstrated in cell culture that the translocated Yops interfere with a variety of host cell functions (5). Some of the Yops, specifically YopE, YopO and YopT, contribute to the disruption of the actin cytoskeleton, either to gain access to non-phagocytic cells or to avoid ingestion by professional phagocytes (6-8). In addition, YopH is a tyrosine phosphatase that inhibits phagocytosis of the pathogen by dephosphorylating proteins in the focal adhesion complex, resulting in the disassembly of this essential complex (9). YopP is known to block the NF*k*B pathway by preventing the activation of I*k*B kinaseβ and abrogating the onset of the pro-inflammatory response (10). YopP also induces apoptosis in infected macrophages (11).

Additionally, YopM has been shown to translocate into HeLa cell nuclei during *Yersinia* infection (12) and it appears to be a necessary virulence factor of *Y. pestis*; however, the function of YopM is still unknown (5).

While much is known about many of the *Y. pestis* and *Y. pseudotuberculosis* virulence factors, little is understood about the global differences in host response to these two pathogens with the exception of our preciously cited report on proteomic characterization of host response to *Yersinia* (13). In this preliminary study, 2-dimensional electrophoresis (2-DE) was used to characterize the soluble protein fraction of U937 cells after exposure to *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis* and several distinctions were identified. Additional characterization of host-pathogen interactions, in particular the human immune response to *Y. pestis* and *Y. pseudotuberculosis,* can provide not only mechanistic information regarding how *Y. pestis* is able to disarm the host immune response resulting in such high lethality, but also supports biomarker discovery efforts for early detection of exposure to *Y. pestis,* a critical challenge in civilian biodefense.

Host-pathogen interactions reflect the co-evolutionary balance that the host and pathogen have reached in order to ensure survival. While microbial pathogens have evolved unique ways to infect human, humans have evolved an elaborate immune system to fight challenges from both viruses and bacteria. Some of the most dangerous and lethal pathogens, such as *Y. pestis*, have also evolved sophisticated mechanisms to thwart the host defense mechanisms. As such, a better understanding of the pathogenic mechanism of *Y. pestis* may lead to better diagnostic capabilities and identify novel therapeutic targets.

Here, 2-dimensional differential gel electrophoresis (2-D DIGE) was used to characterize host response in the monocyte-like cell line, U937, after exposure of *Y. pestis* and *Y. pseudotuberculosis*. The development of the fluorescence-based 2-D DIGE proteomic approach enables the multiplex analysis of multiple proteomic samples within one gel. For example, it is possible to directly compare unexposed macrophages to *Y. pestis* and *Y. pseudotuberculosis* exposed macrophage cells on the same gel, thereby alleviating the complication of gel-to-gel variability. Macrophages cells are primary responders to many bacterial infections and the U937 cell line displays many macrophage characteristics. Thus, this cell type was chosen as an *in vitro* model for proteomic characterization of human immune response to *Yersinia*.

It is recognized that proteins identified from whole cell lysates by two-dimensional electrophoresis (2-DE) are often high-abundant proteins, such as house keeping or structural proteins (14, 15). Less abundant proteins such as transcription factors are difficult to detect due to low cellular concentrations and the overwhelming proteomic signal from higher abundant proteins (15, 16). Complicating matters further, hydrophobic proteins including membrane proteins are notoriously difficult to characterize by 2-DE primarily due to low solubility in buffers used for electrophoresis (17). Therefore, subcellular fractionation was employed here to reduce the proteomic complexity from whole cell lysates and to enrich for subclasses of proteins such as nuclear and membrane proteins. We report differential protein expression profiles of U937 host cells in response to *Y. pestis* and *Y. pseudotuberculosis* exposure, via 2-D DIGE characterization of the subcellular cytoplasmic, membrane and nuclear protein fractions.

Experimental Procedures

Reagents and chemicals

Growth medium RPMI-1640 was purchased from ATCC (Cat.No. 30-2001). Fetal bovine serum was purchased from Invitrogen (Cat. No.16140071). Penicillin-streptomycin solution was purchased from Sigma-Aldrich (Cat. No. P0781). The CyDyes (Cy2, Cy3, and Cy5) and Immobilized Dry-Strips were purchased from Amersham Biosciences.

Human cell culture and bacterial exposure

The human monocyte cell line, U937 (CRL-1593.2) was obtained from ATCC and maintained as frozen stock at –80°C in RPMI-1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin and 10% dimethyl sulfoxide. The U937 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution in a 5% CO_2 incubator at 37°C to log phase (1.2 x 10^6 cells/mL). Cells were then spun down at 800 g for 5 min and suspended in a fresh RPMI-1640 medium without supplements. Ten milliliters of suspension culture containing 3 X 10^7 cells was placed in each cell culture dish (VWR 25382-442). Cells were allowed to attach to the cell culture dish for 2 hours in a 5% CO_2 incubator at 37°C, and cells were then subjected to pathogen exposure.

Y. pestis, Kim5-D27 (2) and *Y. pseudotuberculosis*, PB1 (18), were used. *Yersinia* bacteria were inoculated from frozen glycerol stocks onto a TBA plate and incubated at 26°C for 2 days. A single colony was picked from the TBA plate and streaked onto a TBA medium slant and incubated at 26°C for 2 days. The bacteria were then washed off with 2 mL of 0.033 M $KHPO_4$ for human cell infection. The concentration of bacteria was measured at OD_{620} nm. The reading

was in the range of 5~6 X 10^9 CFU/mL. 1.5 X 10^8 bacteria were directly pipetted into 10 mL of host cell culture containing 3 X 10^7 U937 cells. U937 cells were infected at multiplicity of infection of 5:1 and incubated with bacteria without shaking at 37°C for 4 hours. A negative, mock control exposure was carried out using 0.0033 M $KHPO^4$ without bacteria. After the 4 hour exposure, a final concentration of 100 μg/mL of gentamicin was added to the exposed culture and incubated for 1 hour to kill *Yersinia*. The medium containing the bacteria was then removed by pipette. The U937 cells were washed twice with PBS and then harvested with a cell policeman. The collected cells were centrifuged at 2000 X g for 5 min to remove the residual PBS. The remaining pellets were used for subsequent protein extractions.

Subcellular protein fractionation

Cytoplasmic and nuclear proteins were extracted from the cell pellets described above with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) as described by the manufacture. Membrane proteins were extracted with Mem-PER Eukaryotic Membrane Protein Extraction Reagent kit (Pierce). Halt Protease Inhibitor Cocktail (Pierce) was added to protein extraction reagents prior to extraction at a final concentration of 10 μl/mL to reduce protein degradation. Protein concentrations were measured using the Advanced Protein Assay Reagent (Cytoskeleton Cat# ADV01).

Protein sample cleanup and CyDye labeling

Before CyDye labeling, cytoplasmic and nuclear protein extracts were cleaned using the PlusOne 2-D Clean-Up kit (Amersham). Protein pellets obtained were suspended in labeling buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 20 mM Tris (pH 8.5).

Membrane protein extracts were cleaned using the Perfect-FOCUS kit (Geno Technology). Protein pellets obtained were suspended in the same labeling buffer as above with the addition of 0.5% Triton X-100.

2-D DIGE experimental design

To derive statistically confident data on differential expression, the following experimental design (Table I) was applied. After *Y. pestis* or *Y. pseudotuberculosis* exposure, three cellular protein fractions, cytoplasmic, membrane, and nuclear protein fractions, were extracted. The three cellular fractions were also extracted from the mock, unexposed host cells as the negative control. To determine differential expression in the nuclear protein fraction, 50 μg of pooled internal standard sample, consisting of equal amounts of all nuclear samples, was labeled with Cy2. 50 μg of the control nuclear protein fraction was labeled with Cy3, and 50 μg of each of the experimental nuclear fractions was labeled with Cy5. The internal pooled standard was run together with the Cy3 labeled control protein fraction and the Cy5 labeled protein fractions (from the pathogen exposures) on the same gel. The internal pooled standard was used to normalize protein abundance measurements across the multiple gels to facilitate inter-gel spot matching and relative protein quantitation. The same design was applied for the cytoplasmic protein fractions and membrane protein fractions. To ensure there was an adequate amount of proteins for protein identification by mass spectrometry, 100 μg of unlabeled proteins from the negative control and the pathogen exposed samples were spiked into each gel as shown in Table I.

2-D electrophoresis, gel imaging, and data analysis

Immobiline DryStrips, pH 3-10 NL, 24 cm, were used for the first dimension separation. Isoelectric focusing was carried out on Ettan IPGphor (Amersham) and the following running conditions were used: 30 V rehydration for 12 hours, 500 V for 1 hours, 1000 V for 1 hour, 8000 V for 6.5 hours. Following IEF, the IPG strips were equilibrated for 15 min in equilibration buffer containing 2% SDS, 50 mM Tris-HCI pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 10 mg/ml DTT and then for 15 min in the same buffer except that DTT was replaced with 25 mg/mL iodoacetamide. Equilibrated IPG strips were rinsed and then transferred onto 26 cm x 20 cm 12.5% homogenous polyacrylamide gels. Gels were run using an Ettan DALT 12 at 5 W/gel constant power at 22°C for 30 min and 17 W/gel for 4.5 hours.

Protein detection and relative quantitation of protein expression changes was performed using the DeCyder V4.0 software (Amersham). After electrophoresis, protein gels were scanned using the Typhoon 9410 imager (Amersham). For multiplex analysis, the Cy2 dye was excited at 488 nm and emission spectra obtained at 520 nm, the Cy3 dye was excited at 532 nm and emission spectra obtained at 580 nm, and the Cy5 dye was excited at 633 nm and emission spectra obtained at 670 nm. All gels were scanned at 100 μm resolution. Pooled internal standard and sample gel image pairs from duplicate gel runs were processed with the DeCyder Differential In-gel Analysis (DIA) module to co-detect and quantitate differential protein expression. The Biological Variation Analysis (BVA) module of DeCyder was then used to match all pairwise image comparisons for cross-gel statistical analysis. Comparison of normalized Cy2/Cy3 and Cy2/Cy5 ratios within each gel provided standardized protein abundance for each protein spot. This value was then compared across all gels for each matched spot, and a statistical analysis was performed using the duplicate values from each experimental condition.

Protein spot picking and protein identification

Differentially expressed protein spots with over 1.5 fold changes and with p values ≤ 0.01 were excised directly from 2-D gels either using an automated spot picker (Genomic Solution) or manually. Protein plugs were collected in 200 μL of water in 96-well plates. Protein identities were determined by MALDI-MS and/or LC/MS/MS (Proteomic Research Services, Ann Arbor, MI). The protein spots were subjected to in-gel digestion including reduction and alkylation. MALDI-MS acquisition was performed on an Applied Biosystems Super-DE-STR instrument. Database search was performed using ProFound or MS-Fit for protein identification. When there were insufficient peptides to allow a confident identification by MALDI-MS, LC/MS/MS was performed using a Micromass Q-Tof2 tandem mass spectrometer coupled with nano-LC system. Database search was performed using Mascot. For detailed protocols of protein in-gel digestion and mass spectrometry procedures, see www.proteomicresearchservices.com.

Results

Bacterial exposures

The human monocyte-like cell line, U937, was chosen as an *in vitro* model of the human immune response in macrophages after *Y. pestis* or *Y. pseudotuberculosis* infection. U937 cells grow readily in suspension and can also adhere to the plastic surface of cell culture plates if matrix components such as fetal bovine serum (FBS) are removed from culture. For the purposes of these host-pathogen interaction studies, U937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin until cells reached log phase. Before bacterial exposure, U937 cells were transferred to fresh RPMI-1640 medium without FBS or antibiotics supplements, and allowed to attach to the bottom of the culture dishes for two hours. After the two hour attachment, *Y. pestis* and *Y. pseudotuberculosis* were introduced into the host cell culture at a multiplicity of infection (MOI) of 5:1 (five pathogens to one host cell), and the bacterial exposure was carried out for an additional four hours at 37°C. At the end of the exposure, the medium containing the bacteria was removed, leaving the U937 cells attached to the plastic culture dishes. The host cells were then washed twice with PBS and removed with a rubber policeman. The collected host cells were stored at -80°C until further use for subcellular fractionation.

Subcellular fractionation of cytoplasmic, membrane, and nuclear proteins

The number of proteins expressed in a cell at any given time can reach the tens of thousands, and although 2-DE is capable of simultaneously separating and quantitating thousands of proteins, it is still difficult to characterize the enormous diversity of cellular proteins in any one experiment. Thus, subcellular fractionation was used in this study to reduce proteomic complexity and to

enrich for three subclasses of proteins. Cytoplasmic, nuclear and membrane proteins were extracted from U937 cells by using commercially available fractionation kits with optimization for both cell type and compatibility with 2-DE. Due to volume differences of the cytoplasm and nucleus within a cell and differences in protein abundance within the cytoplasm and the nucleus, $2x10^7$ cells were used for cytoplasmic protein extraction and $1.2x10^8$ cells were used for nuclear protein extraction. Approximately 800 μg of cytoplasmic proteins and 450 μg of nuclear proteins were obtained. $1x10^8$ cells were used for membrane protein extraction and approximately 450 μg membrane proteins was obtained. These protein fractions were then subjected to 2-D DIGE to determine differential expression post bacterial exposure.

Solubilization of membrane proteins for 2-DE

Protein insolubility is a primary reason that membrane proteomic fractions are under-represented by 2-DE characterization, since membrane proteins are poorly soluble in the aqueous rehydration buffers used for isoelectric focusing of the first dimension of 2-DE. A common approach for membrane protein extraction is the use of detergents; however, the detergents used are often unsuitable for 2-DE and therefore must be removed prior to electrophoresis. In this study, membrane protein fractions were extracted using a mixture of detergents in a proprietary membrane protein extraction reagent (Pierce). Several commercially available cleanup kits were then tested for detergent removal and the Perfect-FOCUS kit (Geno Technology) was found to efficiently remove detergents and other non-protein impurities.

In order to counter the solubility challenge of working with membrane proteins, CHAPS, a zwitterionic detergent, has been widely used in electrophoresis buffers to prevent aggregation.

However, the membrane proteins from U937 cells resolved poorly by 2-DE when CHAPS was the sole detergent in the rehydration buffer (data not shown). Significantly, the use of Triton X-100, a nonionic detergent, in combination with CHAPS in the rehydration buffer, improved 2-DE of the membrane proteins (see Figures 1 and 2) resulting in well-separated and well-focused membrane proteins. Therefore, in this study, for characterization of the membrane fraction from U937 cells, the optimized rehydration buffer used for isoelectric focusing contained 4% CHAPS, 0.5% Triton X-100, 7M urea, 2M thiourea, 20 mM Tris, 1% pH3-10 Pharmalyte, 2% Destreak, and 0.0002% bromophenol blue. The second dimension electrophoresis was carried out using standard 12.5% acrylamide gels and SDS buffers.

Identification of differentially expressed protein spots by 2-D DIGE and protein identification by mass spectrometry

2-D DIGE analyses of cytoplasmic, nuclear, and membrane proteins were conducted as outlined in Table I. Following electrophoresis, labeled proteins on the gels were imaged using the Typhoon 9410 imager (Amersham Biosciences), and the images were analyzed using the DeCyder software. In this study, 700 to 800 protein spots were detected across gels. Statistical analysis was performed using DeCyder BVA 4.0 (ANOVA). The experiment was carried out in duplicate, and the data was filtered to retain spots with ANOVA *p* values of 0.01 or less to ensure high statistical confidence of differential expression. The protein expression differences between unexposed control and pathogen exposed samples were compared to determine differential expression. Protein abundance changes over 1.5 were considered upregulated and lower than 1.5 were considered downregulated. Differentially expressed proteins (spots shown in

black circles in Figures 1 and 2) were directly excised from CyDye labeled gels by either manual or robotic spot picking. Proteins were identified by peptide mapping and mass spectrometry. From *Y. pestis* exposed host U937 cells, 7 differentially expressed proteins were identified from the cytoplasmic fraction, 7 from the membrane fraction, and 2 from the nuclear fraction (Table II). From *Y. pseudotuberculosis* exposed host U937 cells, 7 differentially expressed proteins were identified from the cytoplasmic fraction, 2 from the membrane fraction, and 4 from the nuclear fraction (Table III). With the exception of two proteins, the proteins identified from the two pathogen exposures represent proteins that were distinctly differentially expressed after exposure to either *Y. pestis* or *Y. pseudotuberculosis*, and thus the differential expression patterns alone could distinguish the two pathogen exposures. In total, 14 human proteins were identified that were distinctly differentially expressed post *Y. pestis* exposure, and 11 human proteins were identified that were distinctly differentially expressed post *Y. pseudotuberculosis* exposure. The cellular locations, protein names, SWISS-PROT accessions number, gene names, expression fold changes, theoretical pIs, and predicted molecular weights are summarized in Table II and Table III. The known functions of these proteins are listed in Table IV and Table V.

Discussion

A major challenge in modern biology is to understand the expression, function, and regulation of the entire set of proteins encoded by an organism in "normal" or "healthy" states compared to "diseased" states or in response to environmental stimuli. Here, 2-D DIGE was used to detect changes in protein expression in human cells after exposure to *Y. pestis* and *Y. pseudotuberculosis* in an effort to understand the protein expression alterations in the "diseased" state of plague. An advantage of the 2-D DIGE approach is the multiplexing capability whereby up to three protein samples labeled with three fluorescent dyes, Cy2, Cy3 and Cy5, can be co-electrophoresed on a single 2-DE gel. This multiplex approach increases accuracy of protein spot matching between the different samples on different gels of a given experiment. In addition, 2-D DIGE allows the inclusion of an internal pooled standard in each gel for normalization of protein spot intensity across gels. This in turn allows the ratio of relative expression of a protein across an experiment to be compared directly, thereby separating gel-to-gel variation from biological variation.

Notably, it is difficult to simultaneously study all proteins expressed in a given cell because of the large dynamic range in protein expression levels. For example, in human cells, actin is often the most abundant protein having a concentration of 10^8 molecules per cell while regulatory proteins, such as transcription factors found in cell nuclei, may be present at <10 copies per cell (14). Thus, nuclear subcellular fractionation was used to enable the study of low-abundant proteins from the nuclei of U937 cells after exposure to *Yersinia* in order to characterize regulatory proteins that may govern the more obvious differential protein expression noted in these cells after pathogen exposure (Figures 1 and 2).

In addition, analysis of membrane proteins by 2-DE is notably challenging due to low solubility, yet understanding protein changes of receptors on cell membrane surfaces can provide valuable information regarding cell signaling in host immune response. The majority of proteins detected by 2-DE gels from whole cell lysates to date have been hydrophilic in nature (17), and while many efforts have been made to optimize solubility of membrane proteins for 2-DE, there is no universal solubilization method suitable for 2-DE of membrane proteins from all organisms. In this study, it was noted that successful 2-DE of membrane proteins was dependent upon two factors. First, maximizing solubilization of membrane proteins prior to isoelectric focusing was necessary for improved proteomic coverage. Second, maintaining solubility during the first and second dimension electrophoresis steps was critical to well resolved 2-DE gels.

While the detergent CHAPS has been shown to be effective for 2-DE of soluble proteins, it is often ineffective for membrane proteins (17, 19, 20). Here, CHAPS, when used as the sole detergent in the solubilization medium, did not result in good 2-DE separation and was not sufficient to prevent reaggregation of membrane proteins during electrophoresis (data not shown). The addition of Triton X-100 and CHAPS to the solubilization medium, however resulted in improved quality of protein 2-DE separation (Figures 1 and 2). Since membrane proteins play a role in many biological processes including signal transduction, cell adhesion, and transporting processes, the identification of membrane proteins involved in these processes is required in order to fully understand host response to a pathogen. Thus, the improved 2-DE methods for membrane proteins reported here have advanced our ability to characterize host response.

In addition to nuclear and membrane fractions, the soluble cytoplasmic protein fraction of U937 host cells was also characterized. It is well established that *Y. pestis* and *Y. pseudotuberculosis* function via a Type III secretion mechanism to inject the virulence factors into the cytoplasm of host cells (4, 21, 22). Once inside the host cells, these virulence factors are able to manipulate the host defense mechanism resulting in evasion of host immune defense mechanisms. Therefore, the cytoplasmic fraction of U937 cells was also of interest for this study, and results obtained support our earlier 2-DE preliminary characterization of host response from soluble protein fractions (13). In fact, two of the proteins discussed here, macrophage capping protein and monocyte/neutrophil elastase inhibitor, were also detected in the first host response characterization to *Yersinia* (13). Distinction between the proteins identified in these two studies stems from differences in experimental scope where the first study looked at three *Yersinia* species and soluble host proteins, and the current study looked at two *Yersinia* species and three subcellular host proteomic fractions. In addition, improvements in detection and quantitation based on 2-D DIGE capability and the stricter criteria for differential expression, 1.5 fold expression change with a *p* value of 0.01 or less, distinguish the two reports.

With methods in place for nuclear, membrane and cytoplasmic fractionation, a global proteomic study of host response to *Y. pestis* and *Y. pseudotuberculosis* was undertaken.
Previous DNA microarray studies of gene expression changes in host cells have shown that many gene categories are altered by pathogen exposure including genes involved in cell signaling and protein degradation, cytokines and growth factor production, cell adhesion, phagocytosis, apoptosis as well as genes encoding cytoskeletal components (23-25).

Interestingly, we noted some overlap in differentially expressed proteins identified in the present proteomic study and the differentially expressed genes detected from previous genomic studies. While DNA microarray studies can provide insight into the mechanism of pathogenic virulence, mRNA levels may not directly correlate with biological response or protein expression and function. Since proteins are the key elements in biological function, elucidating how host protein expression levels change in response to pathogen exposure is crucial for understanding virulence and pathogenesis. Here, after exposure to *Y. pestis* or *Y. pseudotuberculosis*, differential expression was detected in cytoskeletal proteins, protease inhibitors, proteases, proteins involved in protein turnover, proteins involved in secretion, DNA replication proteins and transcription cofactors, as well as molecular chaperones, as described below.

Cytoskeletal Proteins

In this study, several cytoskeletal related proteins were modulated by *Yersinia* exposure including γ-actin, macrophage capping protein, coronin-like protein P57, L-plastin, α-actinin, ras GTPase-activating-like protein, and spectrin (see Table II and Table III). The actin cytoskeleton is involved in diverse cellular functions such as cell motility, cytokinesis, cellular adhesion, cellular shape, and various signal transduction pathways (6, 26-28). In addition, the actin cytoskeleton system is especially important in lymphocytes and macrophages that are highly motile and can rapidly move to sites of infection and inflammation (29). All of these processes involve spatially controlled assembly and reorganization of the cytoskeletal actin network. Further, the cytoskeleton of host cells is a common target of pathogens (30); and cytoskeletal remodeling has been reported to be a common occurrence due to pathogenic activation (31, 32).

Notably, an essential bacterial virulence factor, *Yersinia* protein kinase A (YpkA), is initially produced in the pathogen as an inactive kinase that is activated upon translocation into the host cell. It has been demonstrated that actin is the cellular activator of YpkA, and the expression of YpkA in cultured epithelial cells was reported to result in complete disruption of the actin cytoskeleton (7).

Of note, actin was downregulated by both *Y. pestis* and *Y. pseudotuberculosis* exposure. Actin-containing microfilaments of non-muscle cells are the most abundant architectural component of the cytoplasm (33). Apart from playing a central role as the basic building block of cellular microfilaments, actin interacts with many actin-binding proteins to form a dynamic cytoskeleton, acting as a platform for many cellular functions. The downregulation of actin by both *Y. pestis* and *Y. pseudotuberculosis* observed in this study, therefore reflects dynamic changes in cellular cytoskeleton remodeling.

The other differentially expressed actin-binding proteins identified here include macrophage capping protein, coronin-like protein p57, L-plastin, α-actinin, ras GTPase-activating-like protein, and spectrin. These actin-binding proteins participate in the formation of the cytoskeletal framework structure and regulate a variety of cytoskeletal properties in response to internal or external stimuli. For example, macrophage capping protein, which was upregulated after *Y. pestis* exposure, is a calcium-sensitive protein that reversibly blocks the barbed rapid growing ends of actin filaments (34). This protein regulates the polymerization of actin filaments and attaches barbed ends of filaments to cellular structures (35), and was also determined to upregulated after *Y. pestis* in our earlier host response study which looked at the soluble host

protein fraction by 2-DE (13). The coronin-like protein p57, which was downregulated after *Y. pseudotuberculosis* exposure, promotes actin polymerization and also interacts with microtubules (36). This protein has been proposed to be involved in the formation of phagocytic vacuoles (37). L-plastin, which was upregulated after *Y. pestis* exposure, is a leukocyte specific actin-binding protein and has been reported to play a crucial role in the host defense mechanism by regulating integrin signal transduction (38). In addition, α-actinin, which was upregulated after *Y. pestis* exposure, is an F-actin cross-linking protein, which is thought to anchor actin to a variety of intracellular structures. This protein is known to play a role in integrin signaling pathway (39). Ras GTPase-activating-like protein, which was downregulated after *Y. pestis* exposure, is a component of Cdc42 signaling to the cytoskeleton. This protein binds to Cdc42 and calmodulin, coupling calcium-mediated calmodulin signaling to cell structure (40). Finally, spectrin, which was also downregulated after *Y. pestis* exposure, is recognized as a component of the cytoskeletal network and has been implicated in several important biological processes involving plasma membranes, including formation and maintenance of cell-cell junctions and regulation of exocytosis (41, 42). It should be noted that only a 70 kDa C-terminal fragment of spectrin was identified in this study.

The upregulation or downregulation of these proteins reflects the remodeling of the cytoskeleton of U937 cells after pathogen exposure. The fold changes in host protein expression likely vary at different timepoints after an exposure; however, in this case, only four hours post pathogen exposure was studied. While the underlying specific mechanisms governing the protein expression changes noted is not entirely clear, alteration in the cytoskeleton of a host cell after a pathogen exposure is a dynamic process and reflects a number of cellular activities. Further, the

identification of differentially expressed cytoskeletal related proteins in U937 cells after *Yersinia* pathogen exposure is in agreement with the numerous reports that the cytoskeleton is the target of *Yersinia* virulence factors, YopH, YopE, YopT and YpkA (5-7).

Proteinase Inhibitors

Monocyte/neutrophil elastase inhibitor, which was significantly upregulated by both *Y. pestis* and *Y. pseudotuberculosis*, is a proteinase inhibitor known to exist at high levels in neutrophils and monocytes (43). This protein efficiently inhibits elastase, cathepsin G, and proteinase-3 (44), and was also determined to upregulated after *Y. pestis* and *Y. pseudotuberculosis* exposures in our earlier host response study which looked at the soluble host protein fraction by 2-DE (13). Interestingly, elastase is a protease that contributes, along with other proteases, to the destruction of bacteria during phagocytosis. In addition, elastase was reported to preferentially cleave virulence factors of *Yersinia*, *Salmonella*, and *Shigellae* (45). Thus, the upregulation of an inhibitor of elastase activity is clearly part of the strategy used by *Yersinia* to evade phagocytosis and other host defense mechanisms.

Proteases

Two differentially expressed serine proteases were also identified in this study. Lon protease-like protein, which was upregulated by *Y. pestis* exposure, is a multi-functional enzyme that is conserved from bacteria to mammalian mitochondria and is known to degrade short-lived regulatory proteins and abnormal proteins including misfolded, unassembled or oxidatively damaged proteins (46-48). Oxidized protein hydrolase, which was upregulated following *Y. pseudotuberculosis* exposure, preferentially degrades oxidatively damaged proteins (49, 50). The

increased expression of these two proteases in response to *Yersinia* exposure implies that degradation of damaged proteins may be part of the host defense and may contribute to host cell survival. Although why the two pathogens affect different protease expression levels is uncertain at this time, it is interesting to speculate that *Y. pestis* has evolved a separate pathogenic mechanism involved in degrading host regulatory proteins. In addition to protein turnover of damaged proteins, the increase in the Lon protease-like protein following *Y. pestis* exposure suggests that some regulatory proteins are altered by *Y. pestis* but not by *Y. pseudotuberculosis*. Whether this is to the benefit or detriment of the host requires further investigation.

Protein Turnover by the Proteasome Pathway

The expression of valosin containing protein p97 was significantly upregulated after *Y. pestis* exposure. This protein is a member of the gene family AAA (ATPase associated with a variety of cellular activities) (51). Valosin containing protein p97, like other members of the AAA family, mediates several important cellular functions in eukaryotic cells, and was reported to be involved in post-mitotic processing of membrane fusions of the endoplasmic reticulum, Golgi apparatus, and nuclear envelopes (52). It has also been reported that valosin containing protein p97 acts as a molecular chaperone that targets many ubiquitinated substrates to the proteasome for degradation (53) including cyclin E, erythropoietin, and cytokine receptors of IL-9 and IL-2, (53, 54). IL-2 and IL-9 are both produced by activated T helper cells after infection and play an important role in host immune responses by promoting the proliferation of activated T-cells and preventing apoptosis. Since biological activities of cytokines are mediated through the binding of

respective cellular receptors, the upregulation of valosin containing protein p97 may contribute to *Y. pestis* evasion of host immune responses via degradation of cytokine receptors.

Protein Secretion

Endoplasmic reticulum protein, which was upregulated by *Y. pestis* exposure, is involved in the processing of secretory proteins within the endoplasmic reticulum (55, 56). Since cytokines are secreted by white blood cells as well as a variety of other cells in response to external stimuli such as bacterial infection, it is interesting to speculate that the increase in this protein is a result of the host immune response against *Y. pestis* and reflects an increased demand of the endoplasmic reticulum protein for the secretion of cytokines. Although characterization of secreted host proteins was not part of this present work, future studies to characterize cytokine expression changes and to analyze secreted host proteins following pathogen exposure should provide a more comprehensive view of the host mounted immune response to *Y. pestis* and *Y. pseudotuberculosis*.

Proteins Involved in DNA Replication, Transcription and Translation

The expression of proteins directly involved in DNA replication, transcription and translation machinery were also altered by *Yersinia* exposure. Specifically, U5snRNP-specific protein, prohibitin and lysyl-tRNA synthetase expression levels were modulated by *Y. pestis* exposure and DNA replication licensing factor MCM3, elongation factor 2, phenylanyl-tRNA synthetase, asparaginyl-tRNA synthetase, and p100 co-activator expression levels were modulated by *Y. pseudotuberculosis* exposure. Following pathogen exposure, expression changes at the mRNAs level for genes or proteins known to be directly involved in transcription and translation have also shown by gene microarray studies (10, 25). The results presented here reflect the fact that

bacteria globally modulate host cellular processes, and support the overall differential protein expression noted after pathogen exposure. The differences between the host responses to the two pathogen exposures studied most likely represents the divergence in virulence and pathogenesis mechanisms between *Y. pestis* and *Y. pseudotuberculosis*, and the fact that these two pathogens affect different host pathways. Additional studies using knockouts in host cells are required to tease out the mechanistic details of alterations in expression levels of proteins involved in DNA replication, transcription and translation; however, it is clear that these alterations are distinct for each pathogen exposure.

Chaperones

Heat shock protein 90-β (HSP90), which was downregulated following *Y. pseudotuberculosis,* is a molecular chaperone that responds in general to cellular stress (57). HSP90 has been reported to interact with tyrosine kinases, some serine/threonine kinases, transcription factors, and cytoskeletal proteins (58). HSP90 was also reported to be associated with survivin, an apoptosis inhibitor. Interestingly, global suppression of this chaperone or disruption of the survivin-HSP90 complex was demonstrated to result in proteasomal degradation of survivin and subsequent apoptosis (59). Thus, the downregulation of HSP90 may reflect increased apoptosis following *Y. pseudotuberculosis* exposure.

Other Proteins

Finally, malate dehydrogenase, which is involved in the citric acid cycle; lamin AC precursor, which is a component of the nulcear membrane; and glyoxalase, an enzyme involved in synthesis of lactoylglutathione, were upregulated following *Y. pestis* exposure. The mechanistic

interpretation of these proteomic alterations is uncertain at this point but it should be noted that the effect of a pathogen exposure is not localized to the immune response. A more in-depth characterization of host response is likely to uncover intricate mechanistic differences between pathogenesis mechanisms within the *Yersinia* pathogens.

Conclusion

In summary, we have demonstrated that *Yersinia* exposures induce marked alterations in protein expression in human monocyte-like U937 cells. Notably, *Y. pestis* and *Y. pseudotuberculosis* induced distinct proteomic alterations in host cells as noted from the proteins identified from cytoplasmic, membrane and nuclear host protein fractions. While two of the identified differentially expressed proteins were detected following exposure to both pathogens, the majority of differentially expressed host proteins were specific to one of the pathogens. From characterization of proteomic host response and interpretation of the changes in protein expression, it is clear that the host proteomic profile represents the interplay between host defense mechanisms, pathogenic virulence, and pathogen evasion of the host immune response. Future host-pathogen interaction studies utilizing additional pathogens, following longitudinal host response over several timepoints, and utilizing more relevant host models such as animals, promise to better define virulence and pathogenesis mechanisms, and to identify potential biomarkers for early detection of pathogen exposure. This type of information could be used not only to combat infectious disease but also from a civilian biodefense standpoint as a diagnostic tool. In support of this approach, the protein expression patterns identified here can distinguish *Y. pestis* exposure from *Y. pseudotuberculosis* exposure representing a step toward presymptomatic detection of infectious diseases. Ultimately, as our knowledge of host-pathogen interaction

increases, so will our understanding of the molecular causes of infectious disease, which will lead to better diagnostics for early detection of diseases as well as novel, next-generation therapeutic approaches to combat infectious diseases.

Acknowledgments

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Figure Legends

Figure1. 2-D DIGE images showing differentially expressed host proteins after *Y. pestis* exposure.

Protein extracts of cytoplasmic fractions C, membrane fractions M, and nuclear fractions N were separated on pH 3-10 non-linear gradient IPG strips and 12.5% SDS-PAGE gels. Differentially expressed proteins identified by mass spectrometry are designated by circles, and the corresponding gene names are shown. Table II shows relative quantitative differences, and Table IV shows protein functions.

Figure 2. 2-D DIGE images showing differentially expressed host proteins after *Y. pseudotuberculosis* exposure.

Protein extracts of cytoplasmic fractions C, membrane fractions M, and nuclear fraction N were separated on pH 3-10 non-linear gradient IPG strips and 12.5% SDS-PAGE gels. Differentially expressed proteins identified by mass spectrometry are designated by circles, and the corresponding gene names are shown. Table III shows relative quantitative differences, and Table V shows protein functions.

Table I. 2-D DIGE Experimental Design

Gel Number	Pathogen Exposures	Protein Fractions	Cy2 Label	Cy3 Label	Cy5 Label	Protein Spike
1	Y. pe	Cytoplasmic	Pooled STD 1	Control	Exposed	Unlabeled control
2	"	Cytoplasmic	Pooled STD 1	Control	Exposed	Unlabeled exposed
3	Y. ps	Cytoplasmic	Pooled STD 1	Control	Exposed	Unlabeled control
4	"	Cytoplasmic	Pooled STD 1	Control	Exposed	Unlabeled exposed
5	Y. pe	Membrane	Pooled STD 2	Control	Exposed	Unlabeled control
6	"	Membrane	Pooled STD 2	Control	Exposed	Unlabeled exposed
7	Y. ps	Membrane	Pooled STD 2	Control	Exposed	Unlabeled control
8	"	Membrane	Pooled STD 2	Control	Exposed	Unlabeled exposed
9	Y. pe	Nuclear	Pooled STD 3	Control	Exposed	Unlabeled control
10	"	Nuclear	Pooled STD 3	Control	Exposed	Unlabeled exposed
11	Y. ps	Nuclear	Pooled STD 3	Control	Exposed	Unlabeled control
12	"	Nuclear	Pooled STD 3	Control	Exposed	Unlabeled exposed

Y. pe = *Y. pestis*
Y. ps = *Y. pseudotuberculosis*
Control = unexposed samples
Pooled STD: Internal standard containing equal amounts of control and exposed protein samples from all 1) cytoplasmic fractions (STD1), 2) membrane fractions (STD2), and 3) nuclear fractions (STD 3).
Protein spike: Unlabeled protein (100 μg) was added to gels to increase total protein concentration for the purpose of spot picking and protein identification by mass spectrometry.

Table II. Identification of Differentially Expressed Proteins after *Y. pestis* Exposure

Protein Fraction	Protein Name	SWISS-PROT Accession #	Gene Name	Fold change* *p* value ≤0.01	Theoretical pI/MW kDa
C	Prohibitin	P35232	PHB	-2.3	5.6 / 29.8
C	γ-actin	P02571	ACTG	-2.0	5.3 / 41.8
C	Endoplasmic reticulum protein	P30040	ERP28	+3.1	6.7 / 29.0
C	Macrophage capping protein	P40121	MCP	+4.5	5.9 / 38.8
C	Lysyl-tRNA synthetase	Q15046	KARS	+2.1	5.9 / 68.5
C	Malate dehydrogenase	P40925	MDHI	+3.1	6.9 / 36.4
C	Monocyte/neutrophil elastase inhibitor	P30740	SERPIN/PI2	+12.6	5.9 / 42.7
M	Valosin containing protein p97	P55072	VCP	+7.1	5.1 / 89.9
M	Acidic leucine-rich nuclear phosphoprotein 32 B	Q92688	ANP32B	+2.9	3.9 / 28.5
M	Lymphocyte L-plastin	P13796	LCP1	+5.5	5.3 / 70.8
M	Lamin A/C precursor	P02545	LMNA	+5.4	6.4 / 65.1
M	Glyoxalase I	Q04760	GLO1	+3.0	5.1 / 20.9
M	Spectrin beta chain	P11277	SPTB	-1.7	5.1 / 246.3
M	Ras GTPase-activating-like protein	P46940	IQGAP1	-1.8	6.1 / 189.9
N	U5 snRNP-specific protein	Q15029	SNRP116	+3.5	4.8 / 110.4
N	Lon protease-like protein	P36776	PRSS15	+4.1	5.9 / 95.6

N, nuclear fraction; C, cytoplasmic fraction; M, membrane fraction.
* Fold change relative to unexposed control expression values.

Table III. Identification of Differentially Expressed Proteins after *Y. pseudotuberculosis* Exposure

Protein Fraction	Protein Name	SWISS-PROT Accession #	Gene Name	Fold change* *p* value ≤0.01	Theoretical pI / MW kDa
C	Monocyte/neutrophil elastase inhibitor	P30740	PI2	+11.4	5.9 / 42.7
C	Oxidized protein hydrolase	Q9P0Y2	OPH	+1.8	5.3 / 81.3
C	P100 co-activator	Q9R0S1	AND1	+1.6	6.6 / 100.4
C	Elongation factor 2	P13639	EF2	+1.6	6.4 / 96.3
C	Similar to EF 2	Q8TA90	EF2*	+1.6	6.5 / 58.2
C	Heat shock protein HSP 90-β	P08238	HSP90B	-2.0	5.1 / 73.2
C	Phenylalanyl-tRNA synthetase β	Q9BR63	PheT	-2.2	6.6 / 66.7
M	α-actinin cytoskeletal isoform	P12814	ACTN1	+1.7	5.3 / 103.2
M	Calmodulin (phosphorylase kinase, delta)	Q13942	CAMIII	+2.4	4.06 / 17.2
N	Asparaginyl-tRNA synthetase	Q43776	NARS	-2.1	5.9 / 63.8
N	Coronin-like protein P57	P31146	CORO1	-2.2	6.3 / 51.7
N	DNA replication licensing factor MCM3	P25205	MCM3	+1.8	5.5 / 90.9
N	γ-actin	P02571	ACTG	-2.6	5.3 / 41.8

N, nuclear fraction; C, cytoplasmic fraction; M, membrane fraction.
* Fold change relative to unexposed control expression values.

Table IV. Function of Proteins that were Differentially Expressed after *Y. pestis* Exposure

Protein Name	Function
Prohibitin	Represses E2F activity, Induces p53 transcription
γ-actin	Cytoskeletal microfilament component
Endoplasmic reticulum protein	Disulfide isomerase, processes secretory proteins within the endoplasmic reticulum
Macrophage capping protein	Binds to actin barbed ends
Lysyl-tRNA synthetase	Lysine tRNA ligase
Malate dehydrogenase	Oxidation of malate to oxaloacetate
Monocyte/neutrophil elastase inhibitor	Fast acting inhibitor of elastase, cathepsin G and protease-3
Valosin containing protein p97	Membrane fusion, ER-associated degradation, cellular chaperone
Acidic leucine-rich nuclear phosphoprotein 32 B	Involved in antigen-mediated cellular responses, leukemogenesis and differentiation
Lymphocyte L-plastin	Cross links actin filaments into bundles
Lamin A/C precursor	Component of nuclear lamina, interacts with nuclear proteins and chromatin
Glyoxalase I	Conversion of methylglyoxal-glutathione conjugate to lactoylglutathione
Spectrin beta chain	Associates with actin and Bind4.1 to form cytoskeleton
Ras GTPase-activating-like protein	Regulates actin cytoskeleton through its interaction with Cdc42, Rac1 and calmodulin
U5 snRNP-specific protein	Splicing mRNA precursors
Lon protease-like protein	Degrades damaged proteins and short-lived regulatory proteins

Table V. Function of Proteins that were Differentially Expressed after *Y. pseudotuberculosis* Exposure

Protein Name	**Function**
Monocyte/neutrophil elastase inhibitor	Fast acting inhibitor of elastase, cathepsin G and protease-3
Oxidized protein hydrolase	Degrades oxidatively damaged proteins
P100 co-activator	Regulates STAT6-mediated transcription
Elongation factor 2	Tranlocates protein chain from the A-site to the P-site
Similar to EF2	Tranlocates protein chain from the A-site to the P-site
Heat shock protein 90-β	Molecular chaperone
Phenylalanyl-tRNA synthetase β	Protein synthesis, phenylalanine tRNA ligase
α-actinin cytoskeletal isoform	Cross links actin filaments into bundles, connects actin fibrils to focal adhesion
Calmodulin	Involved in inflammation, apoptosis, immune response, and cytoskeletal assembly
Asparaginyl-tRNA synthetase	Protein synthesis, asparagine tRNA ligase
Coronin-like protein P57	Promotes rapid actin polymerization
DNA replication licensing factor	Replication initiation factor
γ-actin	Cytoskeleton microfilament component

Figure 1

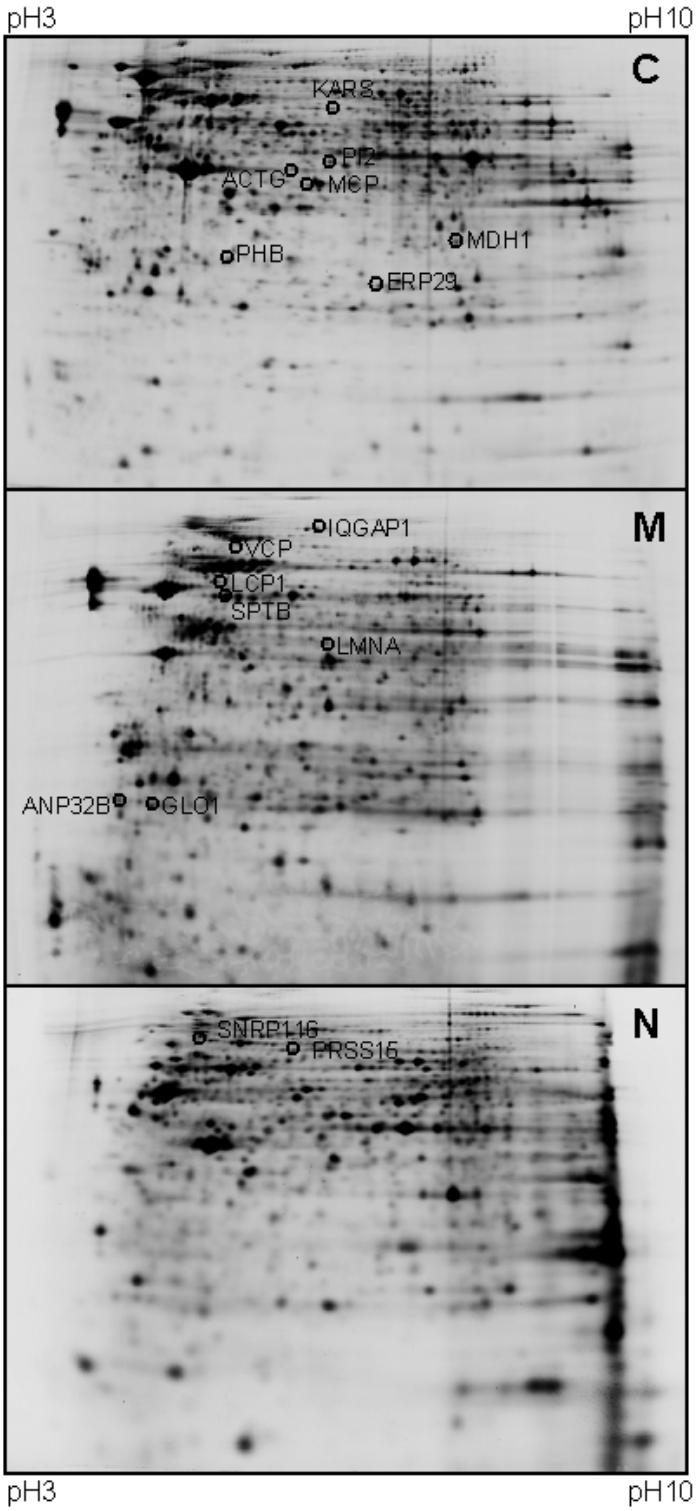
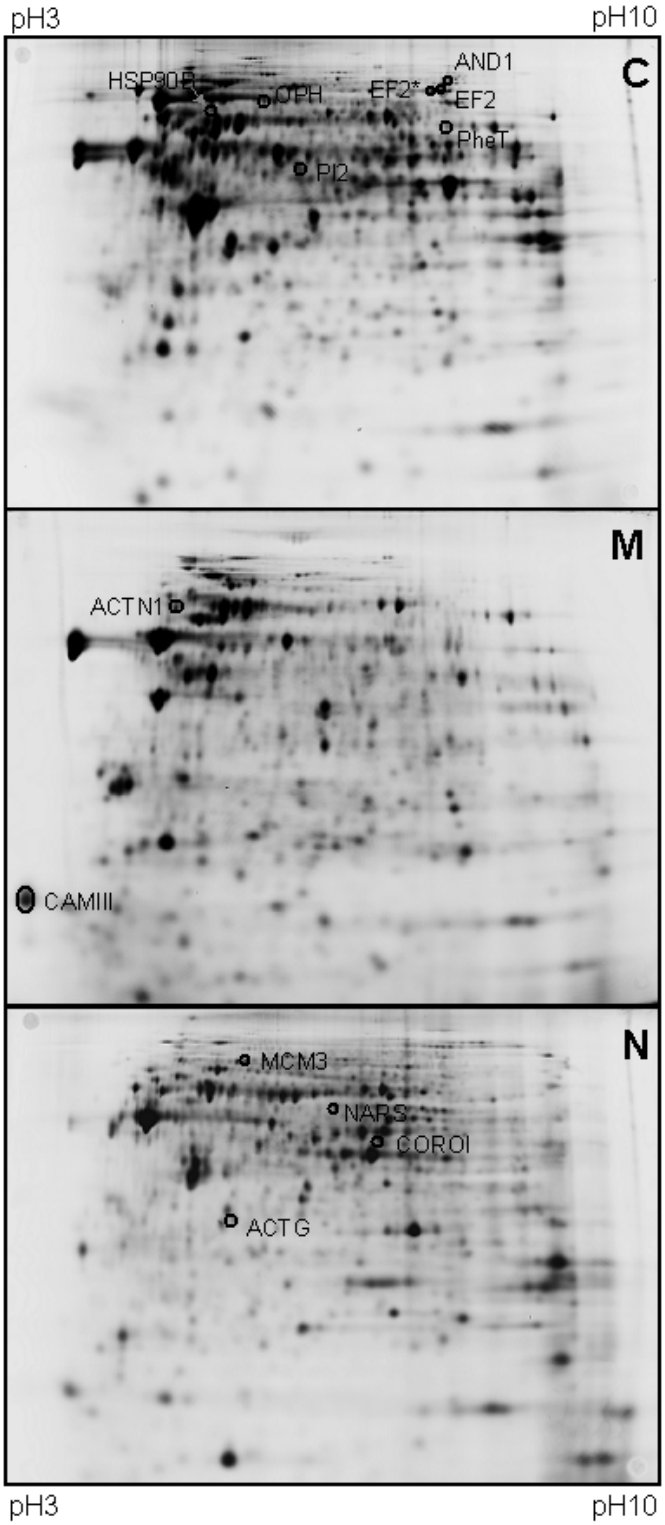

pH3
pH10
C
KARS
PI2
ACTG
MCP
MDH1
PHB
ERP29
M
IQGAP1
VCP
LCP1
SPTB
LMNA
ANP32B
GLO1
N
SNRP116
PRSS16
pH3
pH10

Figure 2


pH3
pH10
C
HSP90B
OPH
EF2*
AND1
EF2
PheT
PI2
M
ACTN1
CAMIII
N
MCM3
NARS
COROI
ACTG
pH3
pH10